To: Jack Kennealy and Ilse Light; ONCOR
From: Gerard Nuovo, MGN Medical Consultants
Re: HPV in situ study
Date:

Dear Jack and Ilse:

I would like to summarize the data/findings that relate to the different issues we discussed recently:

◊ HPV 42, 43, and 44 data. This is summarized in the tables included with this letter. It is clear from this data that high copy HPV 6/11,42,43,or 44 positive cases can cross hybridize with the "high risk" ONCOR probe cocktail (each at 500 ng/ml) at low stringency (1XSSC, 2% BSA, and 45°C for 5 min). Our options are to increase the stringency and/or decrease the concentration of the various probes. To do the latter, we must know the hybridization profiles of the different HPV types.

◊ Cross hybridization profiles of the different HPV types. I performed in situ hybridization using 500 ng/ml of the ONCOR probe cocktails with tissues known to contain a variety of HPV types. The data follows:

Cross hybridization patterns*

	HPV 16	HPV 18	HPV 31	HPV 33	HPV 35	HPV 51
6/11	2+	0	1+	0	weak	0
18	0	3+	0	0	0	
45	0	2+	0	weak	0	0
52	0	0	0	0	2+	0
56	1+	0	1+	0	weak	0

* Done at low stringency.

◊ With this information, we did two sets of experiments:

a) High stringency wash;

b) Relationship of signal for HPV 6/11+ case with different concentrations of HPV 16 probes. I chose HPV 16, of course, because it is the one most responsible for the signal with the HPV 6/11 tissues. The data follow:

A

SIGNAL INTENSITY
Varying stringency

Case	HPV type	specific HPV type	Digene Omniprobe	ONCOR consensus
		Low stringency*		
5	6/11	3+ (Digene)	3+	2+
		High stringency*		
5	6/11	3+ (Digene)	3+	0
17	56	Low stringency		
		3+ (Digene)	3+	3+
17	56	High stringency		
		3+ (Digene)	3+	0

* Low stringency = 5 minutes at 45°C in 1XSSC and 2% BSA
*High stringency = 10 minutes at 62°C in 0.1XSSC and 2% BSA

Obviously, the high stringent conditions eliminate the signal with the HPV 6/11 tissue but also cause us to lose the signal with an important "novel" type - HPV 56.

The next strategy was to see if we could eliminate the signal with the HPV 6/11 tissue at low stringency using a lower concentration of the "culprit" HPV 16. The data follows:

B

HPV 16 concentration study

	500 ng/ml	200 ng/ml	100 ng/ml	50 ng/ml	33 ng/ml	25 ng/ml
6/11 hi*	2+	weak	0	0	0	0
16 hi*	3+	3+	2+	weak	weak	weak
16 low*	1+	1+	1+	0	0	0

* The HPV 6/11 tissue was a cervical low grade SIL with strongest signal I have ever seen. Of course, this was done because if we can eliminate the signal with it, then lower copy HPV 6/11 tissues will also be scored as negative.

In summary, it is clear that a concentration for HPV 16 in the probe cocktail of 100-200 ng/ml should greatly facilitate eliminating the HPV 6/11 cross over.

◊ True blue reagent and hybrisol. I used the hybrisol and true blue for the data just reported. Clearly, these work very well.

◊ Novel types (specifically HPV 39,58,59, and 68). My assistant and I are beginning the experiments using PCR to identify and type new HPVs based on the published endonuclease profiles. I've included in this report a paper which describes the identification of HPV 70. As you can see from the abstract, this type strongly cross reacts with HPV 39,59, and 68. Clearly, if HPV 70 could be included in the ONCOR cocktail, this should permit detection of these types. Hopefully, I will find HPV 70 in my collection. A quick and simple way to enhance the ONCOR system is to prepare several oligos of 40-50 bp from the HPV 70 sequence and include this in the cocktail.

◊ Hybrid capture system. I'll briefly review this information, as it was discussed in my recent Email. The Hybrid capture system is done at high stringency, and one must use >4,000 pg of HPV 6/11 to see a signal with the high risk cocktail. Most HPV 6/11 cervical warts would not have such a high copy number. I think the key to stress is that the ONCOR system is superior to the Hybrid capture system in several ways:

⇒ It detects clinical infection whereas the Digene system also detects subclinical infection;

⇒ Because it is done at low stringency, it can detect a greater range of novel high risk HPV types and still avoid detecting the low risk types by using the correct proportion of HPV types in the cocktail;

⇒ The ONCOR system allows the pathologist to identify the infected cell type (ASCUS, dysplasia, eg) whereas the Digene system does not.

SUMMARY

* The key data is the cross hybridization of HPV 6/11 with the high risk types and the realization that we can eliminate the HPV 6/11 signal by adjusting the concentration of the HPV 16 probe and still maintain low stringent conditions.
* The ONCOR system as it stands now does an excellent job in detecting the "still novel" types (ie, not HPVs 16,18,31,33,35,45,51,52, or 56); these must include HPVs 39,58,59, and 68. It hasn't missed one yet! We should maintain low stringency to continue to make this a selling point of the ONCOR system. The high stringency Digene hybrid capture system can't detect these novel types; this is why they are adding yet more types. The inclusion of HPV 70 (all or part) should allow the ONCOR system to perform even better with most of these "still novel" HPV types.
* If we go to high stringency, we will lose the signal of many of these novel types unless we get probes for all or most of them. Although I'll try to isolate

these novel types, I don't think the ONCOR system needs them to be successful whereas the Digene system does need them.

SIGNAL INTENSITY
High risk types
Low stringency

Case	HPV type	specific HPV type*	Digene Omniprobe	ONCOR consensus
1	16	3+	2+	3+
2	16	weak	1+	1+
3	16	2+	2+	2+
4	16	2+	2+	2+
5	18	3+	1+	3+
6	18	3+	3+	3+
7	31	3+	1+	3+
8	33	3+	3+	3+
9	33	3+	3+	3+
10	35	3+	3+	3+
11	45	3+ (digene)	3+	3+
12	45	2+ (digene)	2+	2+
13	51	2+	2+	2+
14	51	1+	1+	1+
15	52	2+ (digene)	2+	1+
16	52	3+ (digene)	3+	2+
17	56	3+ (digene)	2+	2+
18	56	3+ (digene)	3+	3+
19	novel, 45R	45 = 3+	3+	1+
20	novel, 35R	35 = 1+	1+	1+
21	novel, many types	6,16,31/33/35+	3+	3+
22	novel, 16R	1+ (digene)	1+	1+

* The specific HPV type is from the genomic ONCOR probe except for HPV types 45, 52, novel types, and 56 where the Digene probe was used.
The signal intensity varied from 1+ (weak signal, light blue), 2+ (moderate signal, blue), and 3+ (intense signal, blue-black).

SIGNAL INTENSITY
Low risk types
Low stringency

Case	HPV type	specific HPV type	Digene Omniprobe	ONCOR consensus
1	6/11	3+ (Digene)	3+	1+
2	6/11	2+ (Digene)	2+	0
3	6/11	2+ (Digene)	2+	0
4	6/11	1+ (Digene)	1+	0
5	6/11	3+ (Digene)	3+	2+
6	6/11	2+ (Digene)	2+	0
7	42	2+ (Digene)	2+	0
8	43	1+ (Digene)	1+	0
9	44	3+ (Digene)	3+	1+

NEW DATA

ONCOR consensus probe cocktail data

Low stringency

	Digene probe*	Cocktail 1**	Cocktail 2	Cocktail 3
2	0 (3+ HPV 2)	0	0	0
6/11	3+	2+	0	0
6/11	3+	2+	1+	weak
6/11	3+	1+	0	0
6/11	3+	3+	0	0
6/11	2+	1+	0	0
6/11	1+	0	0	0
6/11	3+	1+	0	0
6/11	1+	0	0	0
6/11	3+	1+	0	0
42	2+	ND	0	0
43	2+	ND	0	0
44	2+	0	0	0
16	1+	1+	1+	1+
16	3+	3+	3+	2+
16	3+	3+	3+	3+
16	2+	2+	2+	1+
16	1+	1+	1+	1+
18	3+	3+	3+	3+
31	2+	2+	2+	2+
31	3+	3+	3+	3+
31	3+	3+	3+	3+
33	3+	3+	3+	3+
45	3+	3+	3+	3+
56	3+	2+	2+	1+
56	3+	2+	1+	1+
still novel	3+ (45 related)	3+	3+	2+
	2+ (16 related)	2+	1+	1+

* The digene probe is their type specific probe.

** Cocktail 1 has 500 ng/ml of HPV 16, cocktail 2 has 200 ng/ml HPV 16, and cocktail 3 has 100 ng/ml. Each cocktail has 500 ng/ml of HPVs 18,33,35, and 51 plus 200 ng/ml of HPV 31.

To: Jack Kennealy, PhD and Ilse Light
From: Gerard Nuovo
Re: HPV testing
Date:

Dear Ilse and Jack:

I am sending some more information with regards to HPVs 45,52, and 56. As you can see, HPV 56 can be detected by HPV 31, HPV 52 is most related to HPV 33, and HPV 45 is most related to HPV 18. You may recall that HPV 31 did detect HPV 56 and HPV 18 did detect HPV 45 when I did these tests using the individual ONCOR genomic probes. The fact that HPVs 31,33, and 18 are in the ONCOR consensus probe cocktail at 500 ng/ml explains why we are detecting these types readily at low stringency. We are a bit lucky that HPV 16, which is causing most of the cross over with HPVs 6/11, appears to be less important in detecting the "novel" types (ie, HPVs 45,52,56), and the "still novel" types of HPVs 39,58,59, and 68.

I look forward to talking with you on Friday.

Sincerely,

Gerard Nuovo, MD

SUMMARY OF PREVIOUS DATA

HPV 16 concentration study

	500 ng/ml	200 ng/ml	100 ng/ml	50 ng/ml	33 ng/ml	25 ng/ml
6/11 hi*	2+	weak	0	0	0	0
16 hi*	3+	3+	2+	weak	weak	weak
16 low*	1+	1+	1+	0	0	0

* The HPV 6/11 tissue was a cervical low grade SIL with strongest signal I have ever seen. Of course, this was done because if we can eliminate the signal with it, then lower copy HPV 6/11 tissues will also be scored as negative.

SUMMARY OF PREVIOUS DATA

Detection frequencies of the different HPV types

	500 ng/ml	200 ng/ml	100 ng/ml	50 ng/ml	33 ng/ml	25 ng/ml
6/11 hi*	2+	weak	0	0	0	0
16 hi*	3+	3+	2+	weak	weak	weak
16 low*	1+	1+	1+	0	0	0

* The HPV 6/11 tissue was a cervical low grade SIL with strongest signal I have ever seen. Of course, this was done because if we can eliminate the signal with it, then lower copy HPV 6/11 tissues will also be scored as negative.

* The digene probe is their type specific probe
** Cocktail 1 has 500 ng/ml of HPV 35, cocktail 2 has 200 ng/ml HPV 16, and cocktail 3 has 100 ng/ml. Each cocktail has 500 ng/ml of HPVs 18,33,39, and 51 plus 200 ng/ml of HPV 31.

To: Jack Kennealy, PhD and Ilse Light
FAX: 301-963-1436
From: Gerard Nuovo
Re: HPV testing
Date:

Dear Ilse and Jack:

I am re-sending the FAX of . Also included with this FAX is an update on the ONCOR probe study; note that I have included HPV 52 and another still novel type.

I look forward to talking with you at 300.

Sincerely,

Gerard Nuovo, MD

NEW DATA (n = 57)

ONCOR consensus probe cocktail data

Low stringency

	Digene probe*	Cocktail 1**	Cocktail 2	Cocktail 3
2	0 (3+ HPV 2)	0	0	0
6/11	1+	0	0	0
6/11	1+	0	0	0
6/11	2+	1+	0	0
6/11	3+	2+	0	0
6/11	3+	2+	1+	weak
6/11	3+	1+	0	0
6/11	3+	3+	0	0
6/11	3+	1+	0	0
6/11	3+	1+	0	0
6/11	1+	0	0	0
6/11	1+	0	0	0
42	2+	-	0	0
43	2+	-	0	0
44	2+	0	0	0
16	1+	1+	1+	1+
16	1+	1+	1+	1+
16	2+	2+	2+	1+
16	weak	1+	1+	1+
16	3+	3+	3+	2+
16	3+	3+	3+	3+
18	3+	3+	3+	3+
31	2+	2+	2+	2+
31	3+	3+	3+	3+
31	3+	3+	3+	3+
33	3+	3+	3+	3+
33	2+	2+	2+	2+
35	3+	3+	3+	3+
35	1+	-	-	1+
51	3+	3+	3+	3+
30	3+	3+	3+	3+
30	3+	3+	3+	3+
30	3+	3+	3+	3+
39	2+	1+	1+	1+
39	2+	2+	2+	2+
39	1+	weak	weak	weak
39	3+	3+	3+	3+
39	3+	-	3+	3+
39	1+	1+	1+	1+
45	3+	3+	3+	3+
45	3+	-	3+	3+
52	3+	3+	3+	3+
52	3+	3+	3+	3+
52	2+	2+	2+	2+
56	3+	3+	3+	3+
56	1+	1+	1+	1+
56	3+	2+	2+	1+
56	3+	2+	1+	1+
58	2+	2+	1+	1+
59	2+	2+	2+	-
68	1+	-	1+	1+
70	3+	3+	3+	2+
70	2+	ND	2+	-
70	1+	1+	1+	1+
still novel	2+ (31 related)	2+	2+	2+
	1+ (18 related)	1+	1+	1+
	1+ (18 related)	1+	1+	1+

Cross homology patterns*
Gerard Nuovo, MD
MGN Medical Research Laboratories

TABLE 2.

	HPV 16	HPV 18	HPV 31	HPV 33	HPV 35	HPV 51	HPV 70	HPV 6/11
6/11	0	0	0	0	0	0	0	3+
16	3+	0	weak	0	0	0	0	0
18	0	3+	0	0	0	0	1+	0
30	0	0	0	0	weak	0	0	0
31	weak	0	3+	0	0	0	0	0
33	0	0	0	3+	0	0	0	0
35	0	0	0	0	3+	0	0	0
39	0	1+	0	0	0	0	1+	0
41	0	0	0	0	0	0	0	0
45	0	1+	0	0	0	0	1+	0
51	0	0	0	0	0	3+	0	0
52	0	0	0	0	0	0	0	0
56	1+	0	weak	0	0	0	weak	0
58	0	0	1+	1+	0	0	0	0
59	0	1+	0	0	0	0	1+	0
68	0	1+	0	0	0	0	1+	0
70	0	1+	0	0	0	0	3+	0

* Done at high stringency (60°C in 0.2XSSC and 2% bovine serum albumin for 10 min) using tissues/Pap smears known to contain the different HPV types listed in the Table. Concentration of each probe at 500 ng/ml.

Gerard Nuovo, MD
MGN Medical Research Laboratories

TABLE 3.

Low stringency

	Digene probe* 810+ HPV31	Cocktail 1**	Cocktail 2	Cocktail 3
2	1+	0	0	0
601	1+	0	0	0
601	1+	0	0	0
601	2+	1+	0	0
601	3+	2+	0	0
601	3+	1+	1+	weak
601	3+	1+	0	0
601	3+	1+	0	0
601	3+	1+	0	0
601	3+	1+	0	0
601	3+	0	0	0
601	3+	0	0	0
601	3+	0	0	0
42	2+	0	0	0
43	2+	0	0	0
44	2+	0	0	0
86	3+	1+	1+	1+
16	3+	1+	2+	1+
16	2+	2+	1+	1+
16	weak	1+	3+	2+
86	3+	3+	3+	3+
86	3+	3+	3+	3+
86	3+	2+	3+	2+
31	3+	1+	3+	3+
31	3+	1+	3+	3+
33	3+	3+	2+	3+
33	3+	3+	2+	3+
35	3+	3+	3+	3+
35	3+	-	3+	3+
51	3+	3+	3+	3+
30	3+	3+	3+	3+
30	3+	3+	3+	3+
30	3+	3+	3+	3+

39	2÷	1+	1÷	1+
39	2÷	2+	2÷	2+
39	1+	weak	weak	weak
39	3+	3÷	3+	3+
39	3+	-	3+	3÷
39	1+	1÷	1+	1÷
45	3÷	3+	3+	3÷
45	3+	-	3÷	3+
52	3+	3+	3÷	3+
52	3+	3+	3+	3+
52	2÷	2÷	2+	2+
56	3÷	3÷	3+	3+
56	1+	1+	1+	1+
56	3+	2+	2÷	1÷
56	3+	2+	1+	1÷
58	2÷	2÷	1÷	1÷
59	2÷	2÷	2+	-
68	1+	-	1+	1+
70	3+	3+	3+	2+
70	2+	ND	2+	-
70	1+	1+	1+	1+
still novel	2+ (31 related)	2÷	2÷	2+
	1÷ (18 related)	1÷	1÷	1+
	1÷ (18 related)	1−	1÷	1÷

* The digene probe is their type specific probe.

** Cocktail 1 has 500 ng/ml of HPV 16, cocktail 2 has 200 ng/ml HPV 16, and cocktail 3 has 100 ng/ml. Each cocktail has 500 ng/ml of HPVs 18,33,35, and 51 plus 200 ng/ml of HPV 31.